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Short Communication

Evaluation of Intracellular Ca^{2+} Concentration by Fura 2 Ratiometry in Encystment-induced *Colpoda cucullus*

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Abstract. In *Colpoda cucullus*, the signaling pathways for encystment induction involving protein phosphorylation have been believed to be triggered by an increase in the intracellular Ca^{2+} concentration promoted by cell-to-cell mechanical contact due to overpopulation. By means of fura 2 ratiometry, the present study showed that the intracellular Ca^{2+} concentration was actually elevated when vegetative cells were induced to encyst by being suspended at a high cell density in the presence of external free Ca^{2+} and suppressed by chelating external Ca^{2+}. This result strongly suggests that an increase in the intracellular Ca^{2+} concentration caused by an inflow of Ca^{2+} promoted by cell-to-cell mechanical contact due to overpopulation enhances the rate of encystation in *Colpoda cucullus*.

Key words: *Colpoda*, Ca^{2+}, encystment induction, fura 2.

INTRODUCTION

Encystment of the ciliated protozoan *Colpoda cucullus* is induced by cell-to-cell mechanical contact due to overpopulation of vegetative cells (Maeda *et al*. 2005) in the presence of external Ca^{2+} (Yamaoka *et al*. 2004, Matsuoka *et al*. 2009). The cAMP concentration (Asami *et al*. 2010; Sogame *et al.* 2011a, b) and phosphorylation level in several proteins were recently shown to be raised (Sogame *et al.* 2011a, b, 2012a; Sogame and Matsuoka 2012) prior to changing protein expression (Sogame *et al.* 2012b) by encystment induction. Both overpopulation-mediated encystment and protein phosphorylation have been reported to be suppressed by the elimination of either external Ca^{2+} by the addition of ethylene glycol tetraacetic acid (EGTA) or intracellular Ca^{2+} by the introduction of ethylene bis (oxy-2,1-phenylenenitrilo) tetraacetic acid (BAPTA) into the cell interior. These results suggest that the signaling pathways for *Colpoda* encystment involving protein phosphorylation and the rate of encystment were activated by the inflow of Ca^{2+}, which was promoted by cell-to-cell mechanical contact due to over population (Sogame *et al.* 2011a).

However, evidence for the elevation of the intracellular Ca^{2+} concentration has not been obtained, although a preliminary assay by means of fura 2 ratiometry (Grynkiewicz *et al*. 1985) was done (Sogame and Matsuoka 2012). Therefore, the objective of the present

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study was to demonstrate, by means of fura 2 ratiometory assays, that the increase in the intracellular Ca^{2+} concentration which enhances the rate of encystations in *Colpoda cucullus* was promoted by cell-to-cell mechanical contact due to overpopulation.

MATERIALS AND METHODS

Cells of *Colpoda cucullus*, the Nag-1 strain, were cultured in a 0.05% (w/v) extract of dried wheat leaves inoculated with bacteria (*Klebsiella pneumoniae*) as food. The bacteria were cultured on agar plates consisting of 1.5% agar, 0.5% polypepton, 1% meat extract and 0.5% NaCl. The *Colpoda* encystment was induced by being suspended at a high cell density (50,000 cells/ml) in 1 mM Tris-HCl buffer (pH 7.2) containing 0.1 mM $CaCl_2$ (Ca^{2+}/overpopulation encystment induction).

The external free Ca^{2+} concentration was raised to 0.1 mM by the simple addition of $CaCl_2$ or was reduced to less than 2×10^{-8} M (in the case of contaminating free Ca^{2+} less than 10^{-6} M) by the addition of 10 µM (final concentration) EGTA to the medium (Fig. 1), to which $CaCl_2$ had not been previously added. The concentration of free Ca^{2+} ($[Ca]_f$) was calculated following the equation reported by Tsien and Pozzan (1989), $[Ca]_f = Kd[Ca]_t/([EGTA] - [Ca]_t)$. Here, Kd represents the dissociation constant (151 nM in pH 7.2) of EGTA for Ca^{2+}, $[Ca]_t$ the concentration of total Ca^{2+}, and [EGTA] the concentration of EGTA.

For the ratiometry assays, 1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy) ethane-N,N,N',N'-tetraacetic acid, pentaacetoxymethyl ester (fura 2-AM) from Dojindo Laboratories was dissolved in dimethyl sulfoxide (DMSO) to give a 5 mM stock solution, and then diluted 1,000 times to produce a test solution with the final concentration of 5 µM containing 0.1% DMSO. Cells cultured for 1~2 days were washed twice in 1 mM Tris-HCl buffer (pH 7.2) by centrifugation (1,500 × g for 1 min.) and suspended for 30 min. in 1 mM Tris-HCl buffer (pH 7.2) containing 10 µM EGTA and 5 µM fura 2-AM for fura 2 loading. The cells were then rinsed twice in three different test solutions (Fig. 1) by centrifugation (1,500 × g for 1 min.), and suspended in three different test solutions (Fig. 1). The relative intracellular Ca^{2+} concentrations were measured with a spectrofluorophotometer as the ratios (F_{340}/F_{380}) of the fluorescence intensities of the cell suspension excited by 340-nm and 380-nm lights according to the method reported by Sogame and Matsuoka (2012). During measurement the cell suspension was stirred to avoid sedimentation of the cells.

RESULTS AND DISCUSSION

Fig. 1 shows the increase in intracellular Ca^{2+} concentration (F_{340}/F_{380} ratio) promoted by cell-to-cell mechanical contact due to overpopulation of *Colpoda* vegetative cells. The F_{340}/F_{380} ratio of the cell suspension was around 2.0 immediately after the vegetative cells were induced to encyst by Ca^{2+}/overpopulation encystment induction. The F_{340}/F_{380} ratio was gradually elevated to 7.2 at 60 min. after the onset of encystment induction (Fig. 1, open circles). When the cells were suspended in 1 mM Tris-HCl buffer (pH 7.2) without the addition of $CaCl_2$ at a high cell density (Fig. 1, closed squares) or at a low cell density together with high-density polystyrene latex particles (PLP), which have been known to induce *Colpoda* encystment by cell-to-PLP mechanical contact (Maeda *et al*. 2005) (Fig. 1, open triangles), the F_{340}/F_{380} ratio tended to be elevated compared to that of cells suspended in it at a low cell density (Fig. 1, closed circles). The F_{340}/F_{380} ratio was hardly increased in the cells suspended at a low cell density in 1 mM Tris-HCl

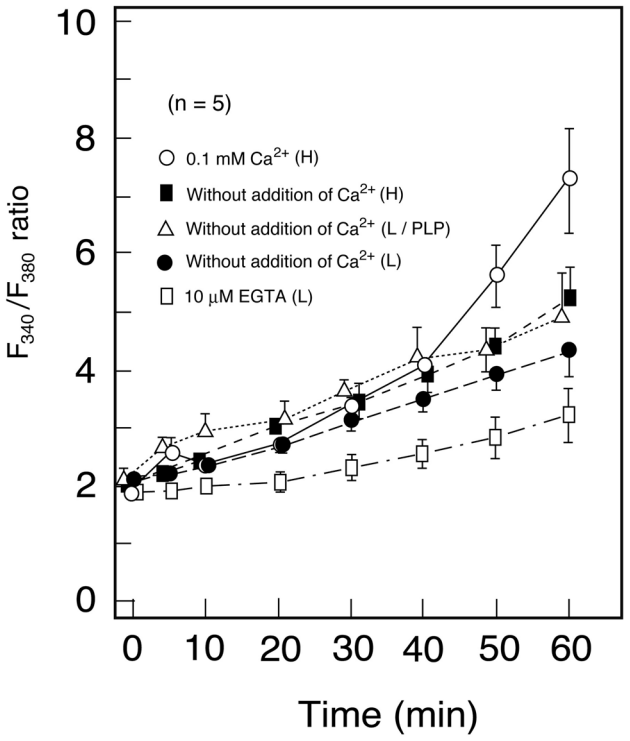

Fig. 1. The elevation in the intracellular Ca^{2+} concentration (F_{340}/F_{380} ratio) in encystment-induced *C. cucullus*. F_{340} and F_{380} are the fluorescence intensities of fura 2 excited at 340-nm and 380-nm light, respectively. The cells loaded with fura 2-AM were suspended in 1 mM Tris-HCl buffer (pH 7.2) containing 0.1 mM $CaCl_2$ (open circles), the same buffer without addition of $CaCl_2$ (closed squares or closed circles), and the same buffer containing 10 µM EGTA (open squares) at high (50,000 cells/ml) ('H') or low (2,000 cells/ml) ('L') cell density. Open triangles show the F_{340}/F_{380} ratios of the cells suspended in 1 mM Tris-HCl buffer (pH 7.2) without addition of 0.1 mM $CaCl_2$ at a low cell density (2,000 cells/ml) together with a high-density (48,000 particles/ml) PLP (26 µm in diameter, Sigma-Aldrich) ('L/PLP'). Points and attached bars correspond to the means of 5 identical measurements and standard errors.

buffer (pH 7.2) in which Ca^{2+} was chelated by the addition of 10 μM EGTA (final concentration) (Fig. 1, open squares). The present fura 2 ratiometry assays (Fig. 1) demonstrated that the elevation in the intracellular Ca^{2+} concentration was actually promoted by cell-to-cell or cell-to-PLP mechanical contact due to overpopulation, and was suppressed by the addition of EGTA. In addition, Ca^{2+}/overpopulation-induced encystment induction and the phosphorylation level in many proteins have been reported to be suppressed by the elimination of either external Ca^{2+} by the addition of EGTA or intracellular Ca^{2+} by the addition of ethylenebis (oxy-2,1-phenylenenitrilo) tetraacetic acid tetra (acetoxymethyl) ester (BAPTA-AM) (Sogame *et al*. 2011a). These results and the present study strongly suggest that the increase in intracellular Ca^{2+} concentration promoted by cell-to-cell mechanical contact due to overpopulation resulted from the inflow of Ca^{2+} from the external medium, and may trigger intracellular signaling pathways for protein phosphorylation. Protein phosphorylation may be responsible for encystment induction.

In contrast to cells that were induced to encyst, the intracellular Ca^{2+} concentration was slightly raised when the cells were not induced to encyst, namely, when the cells were suspended at a low cell density (2,000 cells/ml) in a solution into which $CaCl_2$ was not added (Fig. 1, closed circles). In this condition, protein phosphorylation was slightly enhanced and encystment was slightly induced (Sogame *et al*. 2011a). These results suggest that such spontaneous responses may have resulted from a slight elevation in the intracellular Ca^{2+} concentration caused by the inflow of Ca^{2+} contaminating the external medium. Even in the presence of 10 μM EGTA, a slight elevation of the intracellular Ca^{2+} concentration (Fig. 1, open squares) occurred. If the concentration of contaminating Ca^{2+} in the surrounding medium is assumed to be 10^{-6} M, the addition of 10 μM EGTA (final concentration) would reduce the free Ca^{2+} concentration to 2×10^{-8} M. However, the free Ca^{2+} concentration occurred in the external medium may become much higher than 10^{-6} M because the external medium is further contaminated with Ca^{2+} by the suspension of *Colpoda* cells.

There have been many interesting reports on the membrane flows of Ca^{2+} in some ciliates induced by external mechanical stimuli. For example, membrane flows of Ca^{2+} have been reported to be involved in motor responses of cirri in *Paramecium* (Naitoh and Eckert 1969, Mogami *et al*. 1990) and *Stylonychia* (Mogami and Machemer 1991). In addition, Ca^{2+} has also been reported to be involved in exocytosis (Bilinski *et al*. 1981) as multiple cell signaling molecules in *Paramecium* (Ladenburger *et al.* 2009). On the other hand, in the present study, we demonstrated that the increase in the intracellular Ca^{2+} concentration was actually caused by the inflow of Ca^{2+} from the external medium, which was promoted by cell-to-cell mechanical contact due to overpopulation. Since the increase in the intracellular Ca^{2+} concentration was strongly suggested to be a trigger of the signaling pathways for protein phosphorylation that may be responsible for *Colpoda* encystment induction, further work will be required to conduct a downstream analysis of the event leading to the protein phosphorylation at the molecule level.

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